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Inhibition of Collagen Accumulation in Fibrotic Processes: Review of Pharmacologic Agents and New Approaches with Amino Acids and Their Analogues

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Accumulation of collagen is the major pathologic feature in a variety of fibrotic processes, including dermal fibrosis in progressive systemic sclerosis, morphea, familial cutaneous collagenoma, connective tissue nevi of the collagen type and in keloids. Recent advances in the biochemistry of collagen have allowed us to define specific levels of collagen biosynthesis and degradation at which a pharmacologic intervention can lead to reduced collagen deposition. In this review, we are discussing the mechanisms of action by some of the therapeutic agents currently in use. We further present some new developments involving amino acids and their analogues which could potentially provide us with novel means to reduce the excessive accumulation of collagen in dermal fibrotic processes.

Excessive accumulation of collagen is the major pathologic feature in a variety of clinical conditions characterized by tissue fibrosis. These conditions include localized processes, as for example, pulmonary fibrosis and liver cirrhosis, or more generalized processes, like progressive systemic sclerosis [1–3]. Collagen deposition is a feature of different forms of dermal fibrosis, which in addition to scleroderma, include localized and generalized morphea, keloids, hypertrophic scars, familial cutaneous collagenoma and connective tissue nevi of the collagen type [3–7]. Recent advances in the understanding of the normal biochemistry of collagen [8,9] have allowed us to define specific levels of collagen biosynthesis and degradation at which a pharmacologic intervention could lead to reduced collagen deposition in the tissues. In this overview, we will discuss initially the mechanisms of action by some of the therapeutic agents, many of which are currently in use for treatment of collagen accumulation diseases. We further present some new developments involving amino acids, or their analogues, which interfere with collagen production by isolated cells or tissues under experimental conditions. In the future these compounds could potentially provide us with novel means to reduce the excessive collagen accumulation in diseases.

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Abbreviations:

pC-Collagen refers to a collagen precursor whose amino-terminal extension has been removed while the carboxy-terminal extension is still present on the molecule; the carboxy-terminus is subsequently cleaved by procollagen C-proteinase. Correspondingly, pN-collagen denotes a precursor molecule whose carboxy-terminal extension has been cleaved but the amino-terminal extension remains uncleaved.

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NORMAL BIOSYNTHESIS AND DEGRADATION OF COLLAGEN

The metabolism of collagen in normal tissues is a complex process involving a careful balance between biosynthesis and degradation (Table I). The biosynthetic pathway consists of several steps, many of which are unique to collagen (Fig 1). Many of these steps are also catalyzed by specific enzymes whose activities are stringently modulated by several factors (Table II) [3,10].

The biosynthetic pathway of collagen is initiated by the transcription of the genetic information from a collagen gene to the precursors of messenger RNA (Table I). After extensive processing, including "splicing," "capping," polyadenylation, etc., the functional messenger RNA can serve as a template for translation [3]. The translation of procollagen messenger RNA takes place on the membranes of the rough endoplasmic reticulum of collagen-producing cells, such as fibroblasts. The initial translation product contains a transient peptide sequence, so-called "signal"-peptide, which is removed cotranslationally. After the removal of the signal sequence, the newly-synthesized polypeptide chains of the collagen precursor, procollagen, are vectorially released in the lumen of the rough endoplasmic reticulum. Intracellularly, these polypeptides, known as pro-α chains, undergo extensive co- or posttranslational modifications, and most of these reactions are catalyzed by specific enzymes [1,3,10]; these reactions include the synthesis of 3- and 4-hydroxyproline, hydroxylysine and hydroxylysine-O-glycosides (Table I). The hydroxylated and glycosylated pro-α chains associate, become linked by disulfide bonds, and fold into a triple-helical conformation (Fig 1). This precursor form of collagen, procollagen, contains noncollagenous peptide extensions, so-called propeptides, at both the amino- and the carboxyterminal ends of the molecule. Subsequently, the procollagen molecules are transported through Golgi vesicles into the extracellular space. Here, several additional posttranslational reactions take place: The propeptides are cleaved by at least 2 specific endopeptidases, procollagen N-proteinase and C-proteinase, which remove the amino- and carboxy-terminal peptide extensions from procollagen, respectively. After the removal of the extension peptides, individual collagen molecules align into microfibrillar structures; the association of microfibrils then leads to formation of collagen fibers in the tissues (Fig 1). The fiber structures are stabilized by the formation of covalent lysine- and hydroxylysine-derived intermolecular cross-links. Oxidative deamination of some lysyl and hydroxylysyl residues by lysyl oxidase precedes the formation of these cross-links.

The triple-helical collagen molecules are remarkably resistant to proteolysis by most tissue proteinases, and the removal of collagen fibers in tissues is initiated only by a group of enzymes, known as collagenases (Fig 2). The vertebrate collagenases are metalloproteinases, and they cleave triple-helical collagen molecules at a specific site; the cleavage sequences in interstitial collagens have been shown to be glycine-isoleucine or glycine-leucine. After the initial cleavage by a collagenase, the cleavage products denature at body temperatures and become susceptible to degradation by a variety of proteolytic

TABLE I. *Major steps in the biogenesis and degradation of collagen, their functional significance and modulating factors*

Step[a]	Functional significance	Modulating factors
I. Expression of Genes Coding for Collagen polypeptides		
a. Gene selection	Determines the collagen isotype to be synthesized	Bromodeoxyuridine, environmental factors
b. Transcription	Formation of mRNA precursor	Glucocorticosteroids, possibly other hormones
c. Processing of mRNA precursor	Formation of functional mRNA	None known
d. Translation	Assembly of polypeptide chains	Feed-back inhibition by extension peptides
e. Control of the rate of transcription, mRNA processing or translation	Determines the amount of polypeptides synthesized	Same as Ib and d
II. Intracellular Co- and Post-translational Modifications		
a. Removal of the signal sequence	May be necessary for secretion	Amino acid analogues, such as hydroxynorvaline and canavanine
b. Synthesis of 4-hydroxyproline	Stabilization of triple-helix	Chelators for Fe^{2+}; dehydroproline-containing polypeptides; poly-(L-proline); some divalent cations
c. Synthesis of 3-hydroxyproline	Unknown	Chelators
d. Synthesis of hydroxylysine	Stabilization of covalent cross-links; attachment site for glycosylation	Chelators; some divalent cations
e. Synthesis of hydroxylysine-O-glycosides	May influence cross-link formation and determine the morphology and stability of the fibers	Co^{2+}; free gal-O-hylys; UDP-gal analogues
f. Glycosylation of the extension peptides	Unknown	Tunicamycin
g. Degradation of nonhelical chains	Removal of defective polypeptides and modulation of collagen production	β-agonists; phosphodiesterase inhibitors
h. Chain association and disulfide bonding	Facilitation of the triple-helix formation	S-S reducing agents
i. Triple-helix formation	Pre-requisite for proper secretion	Proline analogues; inhibitors of prolyl 4-hydroxylase
III. Secretion	Transport of procollagen through Golgi apparatus to the extracellular space	Colchicine, cytochalasin B, vinblastine; inhibitors of triple-helix formation; some local anesthetics
IV. Extracellular Modifications		
a. Removal of the extension peptides from procollagen	Necessary for fiber formation	Several amino acids, polyamines, and their analogues; synthetic peptides containing the cleavage sequence; chelators of Ca^{2+}
b. Deamination of certain lysine and hydroxylysine residues	Necessary for cross-link formation	Nitriles; chelators of Cu^{2+}
c. Non-enzymatic glucosylation	May interfere with fiber formation	Glycemic control
V. Fiber Formation		
a. Alignment of the molecules	Formation of microfibrils	Inhibitors of procollagen to collagen conversion
b. Formation of cross-links	Stabilization of the fiber structures	D-penicillamine; inhibitors of lysyl oxidase
c. Supramolecular assembly	Architectural organization of collagen in tissues	None known
d. Interactions with other extracellular macromolecules	Determines the physiologic properties of tissues	None known
VI. Extracellular Degradation		
a. Cleavage by specific collagenases	Rate-limiting step in degradation	Inhibited by chelators of Ca^{2+}, diphenylhydantoin, glucocorticoids, retinoids, α2-macroglobulin, tissue polypeptide inhibitors; activated by sulfhydryl reagents, chaotropic agents, proteolytic activators, monocyte factors
b. Further degradation by peptidases and enzymes metabolizing free amino acids	Removal of degradation products	Chelators of Ca^{2+}; S-S reducing agents

[a] These are the major steps in the sequence they are likely to occur under physiologic conditions *in vivo*; some of the reactions can, however, occur simultaneously or in reverse order (see reference 75).

enzymes [11,12]. The final degradation products, consisting of short peptides and free amino acids, are excreted in the urine or they are metabolized in the liver [13].

From the outline presented above, it is evident that both the biosynthesis and degradation of collagen involve multiple steps, many of which are enzymatically catalyzed (Table I). This situation then presents various levels of collagen metabolism which are potentially amenable to modulation by pharmacologic agents.

PHARMACOLOGIC AGENTS INTERFERING WITH COLLAGEN METABOLISM

Modulators of Transcription and Translation

Since the transcriptional and translational events during the biosynthesis of procollagen are similar to those in general protein synthesis, several agents interfering with protein synthesis at these levels could also modulate collagen production, but most of these agents would not be specific for collagen. There are suggestions that some pharmacologic compounds, such as glucocorticosteroids, tend to exert both catabolic and anabolic actions specific for collagen. Several studies employing tissue explants or intact animals have demonstrated that collagen production is decreased in the presence of glucocorticosteroids [14,15]. The inhibition of collagen has been shown to be more pronounced than the effects on protein synthesis in general. It has been also observed that fluorinated steroids are more potent inhibitors of collagen synthesis than nonfluorinated compounds [16]. These observations would be compatible with clinical findings of dermal atrophy induced by corticosteroid therapy [17,18]. They would also provide an explanation

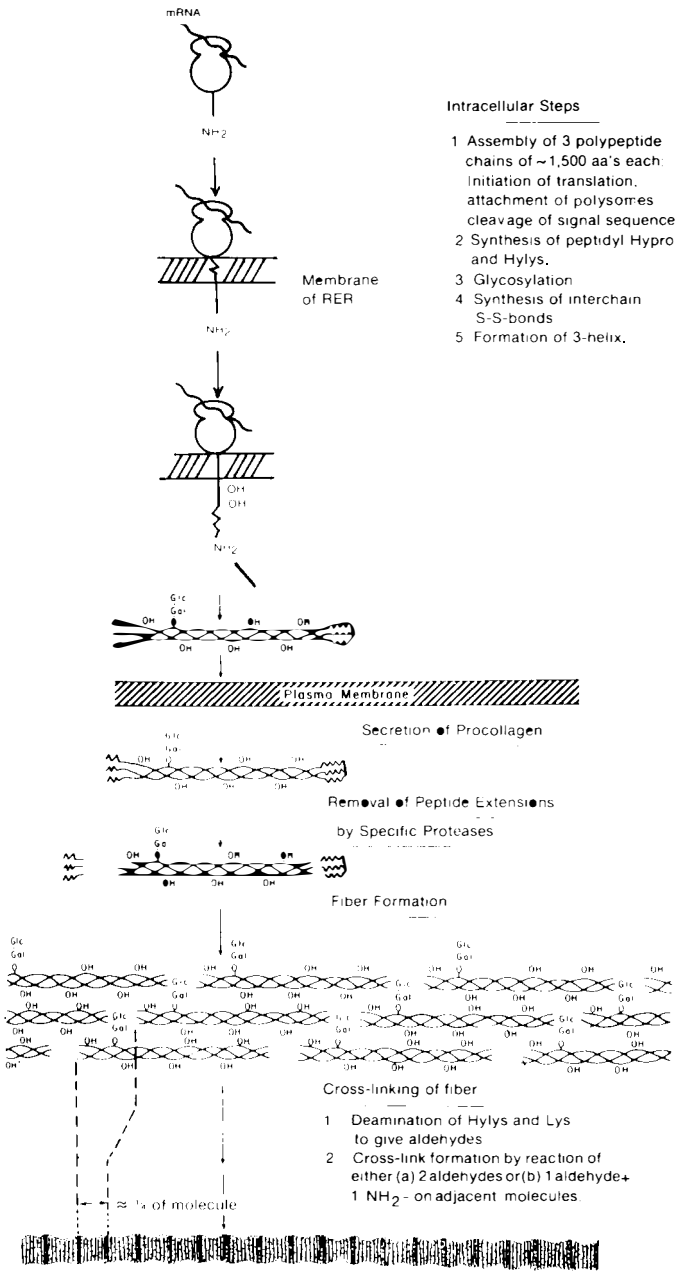

FIG 1. Schematic presentation of the biosynthesis of procollagen and the assembly of the collagen molecules into extracellular fibers. Abbreviation: *mRNA*, messenger RNA; *aa's*, amino acids; *Hypro*, hydroxyproline; *Hylys*, hydroxylysine; *Glc-Gal*, glucosylgalactose attached to a hydroxylysyl residue; *S-S bonds*, disulfide bonds; *lys*, lysine; NH_2 , amino terminal end of the newly-synthesized polypeptide chain, and also the ϵ-amino group of either a lysyl or hydroxylysyl residue (reproduced with permission from Uitto, Ryhänen, and Tan [3]).

for the loss of collagen which accompanies intralesional steriod injection for treatment of keloids and hypertrophic scars [19].

It has been more difficult to define the mode of action of glucocorticosteroids in cell culture systems. In fact, the results from studies employing human skin fibroblast cultures have been contradictory in that both increased and decreased amounts of collagen have been obtained with cells cultured under apparently similar conditions (see reference 20). It appears that the contradictory results can be explained, at least in part, by the experimental tissue culture variables which include the type and concentration of the steroid used. Some trends are evident, however: In general, the halogenated steroids at high concentrations tend to be inhibitory for collagen synthesis. Another general trend has been observed: The studies demonstrating depressed collagen production have mostly

TABLE II. *Characteristics of enzymes participating in the biosynthesis and degradation of collagen*

Enzyme[a]	Substrate[b]	Product	Co-Factors and Co-Substrates
1. Signal peptidase	Nascent pre-pro-α chains	pro-α chains	None known
2. Prolyl-4-hydroxylase	Prolyl residue in pro-gly sequence in pro-α chains*	4-hydroxyproline	O_2, Fe^{2+}, α-ketoglutarate, ascorbic acid
3. Prolyl-3-hydroxylase	Prolyl residue in pro-hyp-gly sequence in pro-α chains*	3-hydroxyproline	O_2, Fe^{2+}, α-ketoglutarate, ascorbic acid
4. Lysyl hydroxylase	Lysyl residue in lys-gly, lys-ser or lys-ala-sequence in pro-α chains*	hydroxylysine	O_2, Fe^{2+}, α-ketoglutarate, ascorbic acid
5. Collagen galactosyl transferase	Hydroxylysine in pro-α chains*	gal-O-hydroxylysine	Mn^{2+}, UDP-galactose
6. Collagen glucosyl transferase	Galactosyl-O-hydroxylysine in pro-α chains*	glc-gal-O-hydroxylysine	Mn^{2+}, UDP-glucose
7. Protein disulfide isomerase[c]	Cystine residues in the extensions of pro-α chains	S-S-bonds stabilizing the correct protein conformation	Thiols
8. Procollagen N-terminal proteinase	Procollagen or pN-collagen	pC-collagen or collagen[d]	Ca^{2+}
9. Procollagen C-terminal proteinase	Procollagen or pC-collagen	pN-collagen or collagen[d]	Ca^{2+}
10. Lysyl oxidase	Lysyl or hydroxylysyl residue in fibrillar collagen	Aldehyde derivatives of lysine and hydroxylysine	Cu^{2+}, O_2
11. Collagenase	Specific sequences in native collagen	Cleavage products TC^A and TC^B	Ca^{2+}
12. Extracellular metalloproteinases	Denatured collagenous polypeptides	Small peptides and free amino acids	Ca^{2+}
13. Hydroxyproline oxidase	Free hydroxyproline	Δ'pyrroline-3-hydroxy-5-carboxylic acid	O_2
14. Hydroxylysine kinase	Free hydroxylysine	O-phosphohydroxylysine	Mg^{2+}, GTP

[a] The action of these enzymes is relatively specific to collagen; the complete sequences of procollagen synthesis and collagen degradation involve additional, less specific enzymes, such as those of transcription and translation.

[b] The reactions indicated by an * are terminated when the pro-α chains fold into the triple helical conformation.

[c] It has not been established whether the formation of interchain disulfides in procollagen involves enzymatic catalysis, as occurs in some other proteins, or whether their synthesis takes place spontaneously.

[d] If intact procollagen is used as a substrate, partially modified products are formed; however, if the partially cleaved proteins serve as substrates, collagen is produced.

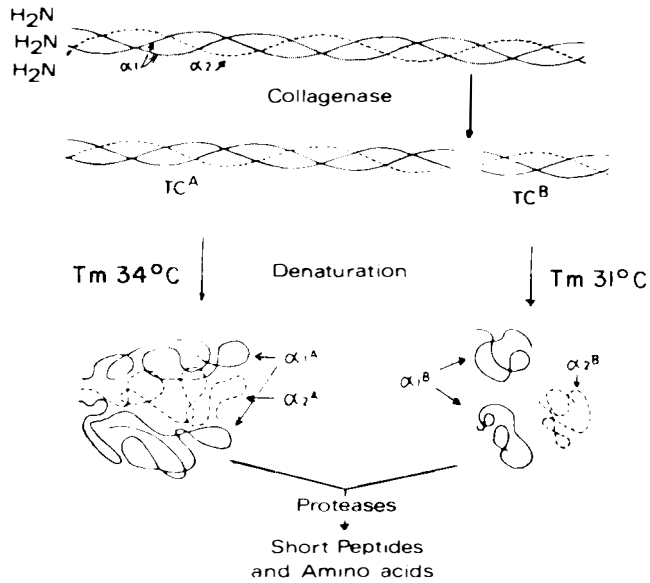

FIG 2. Schematic presentation of cleavage of a collagen molecule by a specific collagenase and subsequent degradation of the cleavage products by other proteases into small peptides and free amino acids. Abbreviations: H_2N-, the amino-terminal ends of collagen α-chains; T_m, the temperature at which the triple helical structure of collagenase cleavage products is denatured into nonhelical peptides. TC^A and TC^B refer to the amino-terminal and carboxy-terminal fragments representing 3/4 and 1/4 lengths of the collagen molecule, respectively, obtained by the specific cleavage with a collagenase; upon denaturation, TC^A and TC^B yield individual polypeptides α^A and α^B (reproduced with permission from Uitto, Ryhänen, and Tan [3]).

utilized adult skin fibroblasts, while those showing enhanced collagen synthesis have employed fetal cells [20].

Careful analyses of the *in vitro* data obtained with human skin fibroblasts in culture do not present any unifying hypothesis or plausible explanation for steroid-induced dermal atrophy. While glucocorticoids appear to suppress collagen production, it is equally feasible that collagen degradation may be enhanced in the presence of steroids [21]. Although the knowledge of collagen degradation under normal conditions is less complete than that of collagen synthesis [22], the glucocorticosteroids appear to depress, rather than enhance, the activity of collagenase in human skin fibroblast cultures [23]. Thus, the variables involved in skin fibroblast cultures do not lend this system amenable to modulation by steroids. Hopefully, a combination of *in vivo* and *in vitro* studies, taking into account the interactions of collagen with other connective tissue macromolecules, may prove more fruitful in understanding the mechanisms of steroid-induced dermal atrophy.

The mechanisms by which other hormones may affect the metabolism of collagen have not been specifically delineated. Nevertheless, these hormones may dramatically alter the amounts of collagen in tissues [13,24,25]. For instance, in the case of hyperthyroidism, the synthesis of collagen is decreased; concomitantly, the degradation of collagen is increased. Hence, the net accumulation of collagen, as a result of these two effects, is depressed. Conversely, in hypothyroidism, the rate of synthesis as well as the rate of degradation is diminished. Parathyroid hormone promotes the resorption of bone which is accompanied by an increase in the degradation of collagen fibers in bone matrix; these effects are opposed by calcitonin. The effects of other hormones are even less well-defined. It has been reported that large doses of estrogen decrease the rate of collagen resorption. Growth hormone, on the other hand, increases collagen synthesis, but because of the remodeling of connective tissues as they enlarge, there is also an increase in collagen degradation. The metabolism of collagen is clearly altered in diabetes, but it is unclear whether the changes are due to insulin or whether they are mediated by fluctuations in glycemic conditions [26,27].

Another feasible control of excessive collagen deposition may evolve from studies demonstrating a feed-back inhibition of collagen production on the translational level. It has been suggested that the extension peptides after their removal from procollagen are capable of inhibiting the synthesis of collagen polypeptides. Specifically, addition of isolated amino-terminal extension peptides of type I procollagen inhibits the synthesis and secretion of procollagen by cultured skin fibroblasts [27]. Furthermore, these peptides, when added to a cell-free translation system producing type I procollagen, depress the synthesis of collagen [28]. Although the details and rationale of this kind of feed-back inhibition are still poorly understood [3], it is possible that synthetic peptide analogues of the procollagen extensions can be developed for pharmacologic use.

Inhibitors of Co- and Post-translational Modification Reactions

During its intracellular biosynthesis, the newly-synthesized procollagen polypeptides undergo several co- and posttranslational modifications which could serve as sites of modulation of the collagen production. In particular, the enzymatic synthesis of 4-hydroxyproline has been shown to be crucial for normal collagen production; with reduced amounts of 4-hydroxyproline, the pro-α chains do not fold into a stable triple-helical conformation at body temperature [30]. Since the triple-helix is prerequisite for collagen secretion at an optimum rate, the nonhelical polypeptides, which are deficient in 4-hydroxyproline, first accumulate intracellularly and are subsequently released into the extracellular milieu at a markedly reduced rate [31,32]. Furthermore, the nonhelical pro-α chains are susceptible to proteolysis by both intracellular and extracellular proteinases. Thus, inhibition of prolyl hydroxylase could reduce the deposition of collagen in tissues.

Prolyl hydroxylase requires ferrous iron for its activity. Based on this observation, in early *in vitro* studies the synthesis of hydroxyproline was inhibited by metal chelators. Extensive studies have been performed, for example, with α,α′-dipyridyl, a chelator which is relatively specific for ferrous iron [30,33]. Although this compound has been proven to be highly effective in limiting the collagen synthesis by isolated tissues or cells *in vitro*, it has not been used in whole animals. It is possible that α,α′-dipyridyl, like other chelating agents, may be toxic due to inhibition of other vital enzymes which are dependent on metal co-factors.

Another approach to inhibit the hydroxylation of prolyl residues has employed divalent cations, such as Zn^{2+}, Pb^{2+} and Co^{2+} (34,35). These metals compete with Fe^{2+} for prolyl hydroxylase, but they are unable to serve as cofactors in the hydroxylation reaction [35]. As a consequence, the procollagen polypeptides are underhydroxylated and retained intracellularly in a similar manner as with α,α′-dipyridyl. In the case of Zn^{2+}, the effective concentrations are in the physiologic range; it has been suggested that the tissue levels of zinc could modulate collagen metabolism and may have significance in pathologic fibrogenesis or play a role in its prevention [35].

An additional promising method to inhibit the synthesis of 4-hydroxyproline in collagen, involves the use of a proline analogue, 3,4-dehydroproline [36,37]. This analogue is readily incorporated into proteins in place of proline and this incorporation can have several consequences. In the case of procollagen polypeptides, it has been demonstrated that the overall hydroxylation is drastically reduced and the pro-α chains remain nonhelical [38]. The inhibition of prolyl hydroxylation by this particular amino acid analogue can be explained by tight complex formation between the analogue-containing polypeptides and prolyl hydroxylase [36]; as a consequence, the enzyme is

rendered nonfunctional. It should be noted that this effect of 3,4-dehydroproline is not shared with other analogues of proline, such as cis-4-hydroxyproline or L-azetidine-2-carboxylic acid, which destabilize the collagen triple helix by mechanisms independent of prolyl hydroxylation (see below). It is conceivable that administration of dehydroproline or dehydroproline-containing small molecular weight peptides could be used in the future to reduce collagen deposition *in vivo*.

Inhibition of Procollagen Secretion

Following the extensive intracellular modifications, the procollagen molecules fold into a triple-helical conformation and are then secreted into the extracellular space [30]. The intracellular translocation of procollagen involves the Golgi apparatus; the protein packaged in Golgi vesicles is transported to the plasma membrane by energy-dependent mechanisms, which can be inhibited by microtubule or microfilament disrupting agents, such as colchicine, cytochalasin B, and vinblastine [30,39]. It is conceivable, therefore, that these agents, or other microtubule disrupting compounds, could be employed for pharmacologic inhibition of collagen accumulation. In fact, colchicine has been used to counteract collagen deposition in animal models of liver cirrhosis and pulmonary fibrosis [40, 41]. Furthermore, it has been suggested that colchicine might be helpful in the treatment of patients with progressive systemic sclerosis [42]. An additional rationale for the use of colchicine in fibrotic processes comes from the demonstration that colchicine increases the release of collagenase by cells [43]. Thus, as a net result of decreased collagen secretion and increased degradation mediated by collagenase, the collagen deposition would be reduced. At this point, however, the experience with the human subjects is rather limited and rigorous documentation of the beneficial effects of colchicine is still lacking.

It is of interest that collagen secretion by cultured cells can also be modified by local anesthetics, such as procaine and lidocaine [44]. In contrast to colchicine, these compounds do not directly interact with microtubules or depolymerize them, but they have been suggested to detach the microtubular system from the plasma membrane [44].

Inhibitors of Collagen Cross-linking

Following the secretion, the procollagen molecules are converted proteolytically to collagen by the removal of extension peptides (see below). The collagen molecules then align in a fibril structure in a characteristic quarter-stagger arrangement (Fig 1). The fibril structures are subsequently stabilized by the formation of covalent cross-links [45]. In the absence of cross-linkage, the fibers lack the tensile strength necessary for functional connective tissues. In addition, the uncross-linked collagen molecules are more susceptible to degradation by collagenases [46]. Thus, in the absence of cross-link formation, the net deposition of collagen is diminished.

The synthesis of collagen cross-links is initiated by the conversion of some lysine and hydroxylysine residues to corresponding aldehydes. This reaction is catalyzed by lysyl oxidase, a copper-requiring extracellular enzyme [47,48]. The activity of lysyl oxidase is readily inhibited by nitriles, such as β-aminopropionitrile, and these compounds have been widely tested in several animal models of tissue fibrosis. The results indicate that the accumulation of collagen is successfully prevented by these compounds. Additionally, encouraging preliminary results have been obtained in limited clinical trials in patients with urethral strictures, tendon adhesions and pulmonary fibrosis (see reference 49). The usefulness of the nitriles in generalized fibrotic conditions, such as progressive systemic sclerosis, remains to be tested.

Another pharmacologic agent which interferes with collagen cross-link formation is D-penicillamine. Its mode of action in cross-linking is varied; first, D-penicillamine is a chelator of copper and thus reduces the activity of lysyl oxidase. It has also been shown that copper is required for the synthesis of lysyl oxidase protein, and in copper deficiency, less lysyl oxidase is functional in tissues [50]. Secondly, it has been shown that D-penicillamine is able to complex with lysine-derived aldehydes making them unavailable for cross-link formation [51]. As a net result of these effects, newly-synthesized collagen remains soluble and is degraded more rapidly, thus contributing to reduced collagen content in tissues. D-penicillamine has been employed for treatment of collagen accumulation diseases, such as hepatic fibrosis, progressive systemic sclerosis, and morphea. In the case of both systemic and localized scleroderma, some success has been reported [52,53], but the evaluation of the full benefits of D-penicillamine in these conditions would require controlled clinical trials with larger patient population.

Modulation of Collagen Degradation

The net amount of collagen in tissue is a balance between synthesis and degradation. Although in most fibrotic conditions, such as progressive systemic sclerosis, the collagen deposition is due to increased synthesis rather than decreased degradation, in a case of localized plantar fibromatosis, the collagen accumulation has been shown to result from reduced collagenase activity [54]. In this and other fibrotic conditions, it would thus be helpful to induce collagenase activity which would facilitate the removal of excess collagen. Unfortunately, no pharmacologic agent has been successful as yet in increasing the collagenase activity *in vivo*. However, under *in vitro* conditions, several factors, including sulfhydryl reagents, chaotropic agents and proteolytic treatments, have been shown to activate preexisting latent collagenase [9,55]. In addition, an immunologic factor, purified from monocyte cultures, has been demonstrated to stimulate the production of collagenase by cultured synovial cells [56]. It is conceivable, therefore, that these developments could form a basis for collagenase induction by pharmacologic means and such agents would be beneficial for patients with fibrotic processes.

In contrast to collagenase induction and activation, this enzyme can be readily inhibited by several compounds, some of which are important in the treatment of human diseases. In particular, diphenylhydantoin has been shown to be beneficial in treatment of patients with recessive dystrophic epidermolysis bullosa, and its clinical efficacy has been suggested to involve inhibition of collagenase [57]. The drug is probably not a direct inhibitor of collagenase activity, but it reduces the production of enzyme protein by fibroblasts [57]. It should be noted that diphenylhydantoin may have multiple effects on collagen metabolism, and thus its effects on connective tissue are probably complex.

In addition to diphenylhydantoin, other pharmacologic agents have been shown to suppress the production of collagenase in tissue culture situations. For example, glucocorticosteroids, such as hydrocortisone or dexamethasone, are capable of inhibiting collagenase in human skin explant cultures [23,58]. In contrast, some studies have suggested that collagen degradation may increase in the presence of glucocorticosteroids [29]. Nevertheless, it is currently unknown if any change in collagenase activity does occur in patients undergoing treatment with glucocorticosteroids.

More recently, it has been demonstrated that retinoids, both naturally occurring tretinoin and synthetic isotretinoin, suppress the activity of collagenase in synovial cell cultures [59]. It was postulated that the inhibition was due to suppression of the synthesis of collagenase protein. Similar inhibition of collagenase activity has also been noted in human skin fibroblast cultures, but here the inhibition was suggested to be due to increased synthesis of an inhibitor of collagenase activity [23]. Nevertheless, in the future, retinoids may provide a means to

modulate collagenase activity in patients whose disease involves abnormalities in collagen degradation.

MODULATION OF COLLAGEN PRODUCTION BY NEW APPROACHES WITH AMINO ACIDS AND THEIR ANALOGUES

Inhibition of Collagen Production By Proline Analogues

An interesting and potentially specific way to interfere with collagen accumulation in tissues would be intracellular inhibition of the triple-helix formation. One of the recent developments involves the use of proline analogues for this purpose.

Several structural analogues of proline, *e.g.*, *cis*-4-hydroxy-L-proline (*cis*-hydroxyproline), L-azetidine-2-carboxylic acid (azetidine carboxylic acid), 3,4-dehydroproline, *cis*-4-bromo-L-proline, and *cis*-4-fluoro-L-proline, have been shown to be incorporated in place of proline into proteins (see reference 38). The incorporation of these analogues into polypeptides can dramatically alter the structure and metabolism of the protein. Incorporation of the analogues into collagen, a protein relatively rich in imino acids, proline and *trans*-4-hydroxy-L-proline (hydroxyproline), has been demonstrated to have several consequences: For example, the newly-synthesized polypeptides of procollagen do not fold into a stable triple helical conformation if a critical number of prolyl residues has been replaced by the analogue (Fig 3) [60,61]. Since the triple helical conformation is a prerequisite for normal secretion of procollagen, the non-helical pro-α chains are extruded from the cells into the extracellular milieu at a markedly reduced rate [62]. The nonhelical polypeptides are also susceptible to degradation by intracellular as well as extracellular proteinases which do not degrade triple-helical collagen. Consequently, the net production of extracellular collagen is decreased in the presence of the analogues (Fig 3) [63] . Recent studies have also demonstrated that incubation of 17-day-old chick embryo tendon fibroblasts in primary culture with *cis*-hydroxyproline results in an inhibition of growth of these cells [64]. Subsequently, it was also shown that this analogue retards the spreading and proliferation of normal murine connective tissue cells, but not of their counterparts rendered tumorigenic by viral, chemical, or spontaneous transformation. [65] On the basis of the latter observations, it was suggested that the inhibition of the growth of normal cells by the analogues could be explained by the deficient production of extracellular collagen which may be required to support cell spreading and growth.

We have recently extended the previous observations made on the proline analogues in animal systems to human skin fibroblast cultures. The experiments were designed to provide answers to the following questions:

1. Is the production of procollagen reduced in the presence of the analogues, and if so, does the inhibition occur on the transcriptional, translational, or posttranslational level?
2. Do the analogues interfere with the attachment and proliferation of cells, and if so, is the effect dependent on reduced collagen production by the cells?
3. Could proline analogues potentially serve as pharmacologic agents limiting collagen deposition in various forms of dermal fibroses?

The results indicated that azetidine carboxylic acid markedly reduced the production of extracellular procollagen by normal skin fibroblasts. Examination of the procollagen production by the cells at different levels of protein synthesis and degradation indicated that the site of inhibition of collagen production occurred at the posttranslational level. Specifically, the transcription of the procollagen genes into mRNA was not affected, since the mRNA activity, measured by cell-free translation in a rabbit reticulocyte lysate system, was the same in cells incubated without and with the analogue [66]. Also, the translational rate of procollagen polypeptides in the cells was only slightly decreased, if the values were corrected for changes in the specific radioactivity of prolyl-tRNA complexes, the immediate precursor of peptide-bound proline in the molecule [67]. In contrast, significant increases in the degradation of newly-synthesized collagen polypeptides, measured as the fraction of {^{3}H}hydroxyproline in dialyzable form, were noted [66]. The increased posttranslational degradation of the analogue-containing peptides, which have been previously [38,61] shown to be in a nonhelical conformation, probably reflects intracellular degradation of collagen. As a consequence, the net production of extracellular collagen is inhibited (Fig 3).

In further studies, we examined the effects of 2 of the proline analogues, *cis*-hydroxyproline and azetidine carboxylic acid, on the growth characteristics of human skin fibroblasts in culture [66]. The results indicated that the proliferation of the cells was markedly reduced in the presence of the analogues. The attachment of the cells on the plastic substratum was also decreased, perhaps explaining, at least in part, the compromised growth potential. The inhibition of cell growth was achieved with analogue concentrations which were considerably lower than those affecting the collagen metabolism. Thus, the compromised growth potential is probably not a consequence of reduced collagen production, as has been suggested previously [65]. Rather, the reduction in the rate of proliferation and attachment of the fibroblasts may reflect altered functions of other pericellular or cell membrane proteins which have incorporated the analogue.

In these studies, we have also cultured fibroblasts from the skin of patients with active scleroderma; previously these cells have been shown to produce procollagen at an increased rate [68,69]. Incubation of the scleroderma fibroblasts with azetidine carboxylic acid reduced the production of extracellular procollagen as was observed with control cells [57]. Recent studies, employing animal models of pulmonary fibrosis and liver cirrhosis, have demonstrated the effectiveness of *cis*-hydroxyproline and azetidine carboxylic acid in reducing the accumulation of collagen when administered systemically [70,71]. It is conceivable, therefore, that development of proline analogues could potentially provide us with the means to reduce the excessive accumulation of collagen in human tissues.

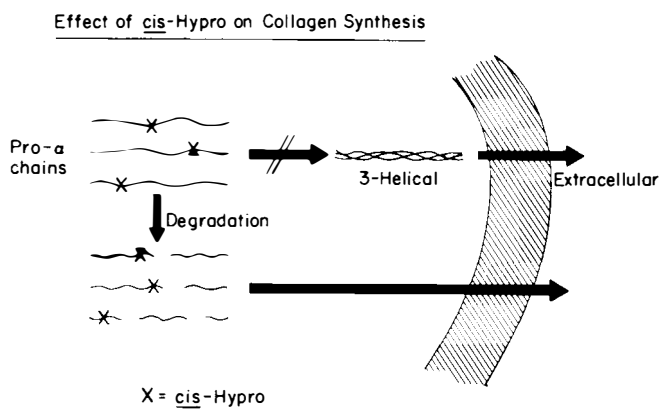

FIG 3. Schematic presentation of how *cis*-hydroxyproline and other proline analogues may reduce the production of extracellular collagen. The newly synthesized pro-α chains of procollagen containing the analogue (*X*) are unable to fold into a stable triple helical conformation and, therefore, are not secreted into the extracellular milieu at a normal rate. Instead, the nonhelical pro-α chains are degraded, and the short breakdown products, even after transport into the extracellular space, are unable to form collagen fibers.

Inhibition of the Conversion Of Procollagen to Collagen by Amino Acids, Polyamines, or Their Structural Analogues

Another level for potential inhibition of collagen deposition in tissues is the conversion of procollagen to collagen. This conversion is a prerequisite for proper alignment of the collagen molecules to form fibers and for subsequent stabilization of the fiber structures by covalent cross-links (Fig 1). It has been

recently shown that arginine and its analogue, canavanine, inhibit the removal of the carboxy-terminal extension from type I procollagen [72]. As an extension of these observations, we have recently examined the effects of a variety of amino acids, polyamines, and their structural analogues on the conversion of type II procollagen to collagen using pulse-chase techniques [73]. The results clearly demonstrate that several amino acids interfere with the conversion of type II procollagen to collagen in short-term organ cultures (Fig 4). Specifically, β-alanine, arginine, asparagine, glutamine, hydroxylysine, lysine and ornithine inhibit the removal of carboxy-terminal extension from type II procollagen. We further demonstrated that several naturally occurring polyamines, such as agmatine, cadaverine, putrescine, and spermine likewise inhibit the conversion of pC-collagen to collagen. In addition, a lysine analogue, S-2-aminoethylcysteine, as well as canavanine, are inhibitory. Finally, ε-aminocaproic acid, a well known inhibitor of proteinases causes the accumulation of pC-collagen in tissues [73].

The common structural feature of all the inhibitory compounds is the presence of a free terminal amino group or amide. It is clear, however, that an α-amino group is not a sufficient structural feature to make a compound an inhibitor, since several α-amino acids, including alanine, glycine, isoleucine, leucine, phenylalanine, threonine, tryptophan, and valine, were not effective in our test system.

The mechanisms of the inhibition of procollagen conversion by the compounds tested by us are not entirely clear at present. It has been suggested that arginine might prevent the removal of the carboxy-terminal extension by interfering with the aggregation of procollagen molecules [72]. Alternatively, there could be a direct inhibition of the procollagen C-proteinase. In our study, we were able to inhibit the removal of the carboxy-terminal extension with several compounds, including ε-aminocaproic acid, a well known proteinase inhibitor. These results thus suggest that the interference with conversion may be a result of direct inhibition of procollagen C-proteinase.

The results of these studies suggest novel approaches to control the collagen deposition in pathologic fibrotic conditions. Potentially, administration of nontoxic compounds containing the appropriate structural features and available in a form which would allow achievement of effective tissue concentrations would lead to accumulation of pC-collagen. Since pC-collagen is not capable of forming fibers of ordinary tensile strength, these poorly cross-linked molecules would probably be degraded by tissue collagenases at an accelerated rate. Similar approaches have been recently suggested by the use of synthetic peptides which interfere with the enzymatic conversion of procollagen to collagen [74].

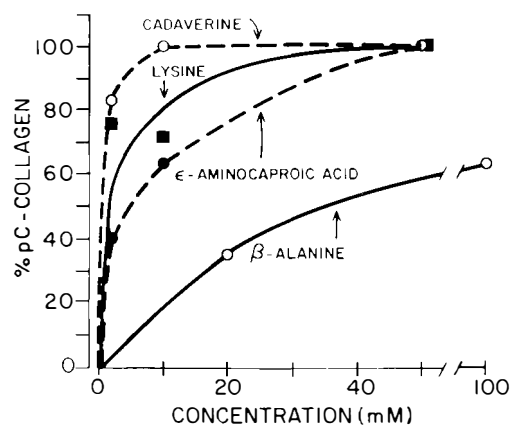

FIG 4. Inhibition of conversion of type II pC-collagen to collagen by varying concentrations of cadaverine, lysine, ε-aminocaproic acid, and β-alanine. 17-Day-old chick embryo sterna were pulse-labeled for 30 min with [^{14}C]proline, and the protein synthesis was inhibited by the addition of unlabeled proline and cycloheximide. The test compounds were then added in concentrations indicated; at the end of the 180-min chase period, the [^{14}C]proteins were examined by SDS-polyacrylamide gel electrophoresis and quantitated by scanning the fluorographs, as described in reference 73. The relative distribution of pC-collagen is expressed as [^{14}C]pC-α-chains as percent of the sum of [^{14}C]pro-α, [^{14}C]pC-α, [^{14}C]pN-α, and [^{14}C]α-chains. Symbols: ○---○, cadaverine; ■—■, lysine; ●---●, ε-aminocaproic acid; ○——○, β-alanine (reproduced with permission from Ryhänen et al [73]).

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